

Reversal of the Antimicrobial Activity of Trimethoprim by Thymidine in Commercially Prepared Media

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Received for publication 30 July 1971

The ability of a potent dihydrofolate reductase inhibitor, trimethoprim, to inhibit the growth of *Escherichia coli* B in vitro is dependent on the composition of the medium in which the cells are grown. The inhibition observed in minimal broth could be partially reversed by the addition of thymidine, ribonucleosides, amino acids, and vitamins. No reversal occurred in the absence of thymidine. In a number of commercially prepared media, the inhibitory activity of trimethoprim correlated inversely with the amount of thymidine found to be present by microbiological assay. The significance of these findings for the routine testing of new, synthetic antibacterial agents is discussed.

Trimethoprim, 2,4-diamino-5-(3',4',5'-trimethoxybenzyl) pyrimidine, is a synthetic, broad-spectrum, antimicrobial agent which acts as a specific inhibitor of the dihydrofolate reductase of bacteria. The major consequence of this inhibition is a drastic and rapid reduction in the level of tetrahydrofolate and its cofactors that are required by the bacterial cell for the synthesis of purines, pyrimidines, and certain amino acids and for the initiation of protein synthesis (6, 9).

When tested in vitro, the ability of trimethoprim to inhibit various organisms is dependent upon the medium in which the test is performed (3, 5). These observations suggested that the medium might be the exogenous source of metabolites whose normal de novo synthesis was blocked by the action of trimethoprim. This hypothesis is not unreasonable since the normal end products of the folic acid pathway are purines and pyrimidines, and "salvage" pathways for these compounds are known to be present in microorganisms (9). Once assimilated by the cell, these compounds would be expected to bypass, in a noncompetitive manner, the inhibition caused by trimethoprim.

In this study, we investigated the role of end products of folate metabolism as potential antagonists of the activity of trimethoprim. Evidence was obtained that thymidine is the metabolite essential for this effect. In addition, it is shown that the degree of activity of trimethoprim in a particular commercial medium may be correlated with the concentration of thymidine it contains.

MATERIALS AND METHODS

The source of each of the commercially prepared media is given in Table 2. The minimal broth contained (in grams per liter): glucose, 5.0; NaCl, 1.0; KH_2PO_4 , 4.2; K_2HPO_4 , 8.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00025; CaCl_2 , 0.005; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.025; Na_2SO_4 , 0.1; and NH_4Cl , 0.8. The amino acid supplement contained (in milligrams per liter): L-aspartic acid and L-glutamic acid, 50.0; L-leucine, 10.0; 18 other amino acids, each 20.0. The nucleic acid base (riboside) supplement contained (in milligrams per liter): adenosine, guanosine, and cytidine, each 20.0; uridine, 10.0. The vitamin supplement contained (in milligrams per liter): *p*-aminobenzoic acid, calcium pantothenate, thiamine, niacinamide, pyridoxine, pyridoxamine, and folic acid, each 1.0; biotin, 0.1; and vitamin B_{12} , 0.01. Minimal broth to which amino acid, nucleic acid base, and vitamin supplement had been added is identified as "supplemented" broth.

Chemicals. Thymidine was purchased from Sigma Chemical Co., St. Louis, Mo., or Schwartz Bio-Research, Inc., Orangeburg, N.Y. All other nucleosides were bought from Sigma Chemical Co. Amino acids and vitamins were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, Calbiochem, Los Angeles, Calif., or Sigma Chemical Co. Casein hydrolysate was obtained from General Biochemicals, Chagrin Falls, Ohio. Trimethoprim [2,4-diamino-5-(3',4',5'-trimethoxybenzyl)pyrimidine] was synthesized previously in this laboratory (13) and dissolved by heating in dilute acid.

Organism. *Escherichia coli* B was obtained from R. Greenberg, University of Michigan. Stock cultures were maintained on nutrient agar (Difco) slants and transferred monthly.

Growth of *E. coli* B in the presence of trimethoprim. Reversal of the antimicrobial activity of trimethoprim

was studied by measuring the effect on growth of various supplements added to minimal medium containing trimethoprim (10 $\mu\text{g}/\text{ml}$). Optically calibrated tubes were prepared containing 2.5 ml of double-strength minimal medium; supplements were added, and the mixture was brought to 5.0 ml with distilled water. The bacterial inoculum was prepared as follows. *E. coli* B was grown at 37 C in supplemented broth, and subcultured on minimal broth. On the following day, the organism was again transferred to minimal broth and allowed to grow for 5 to 6 hr. The organisms were washed twice and resuspended in saline to give a density of 110 Klett units at 660 nm (ca. 10^8 organisms/ml) and then further diluted so that each tube received 5×10^4 organisms (0.1 ml). Controls for the initial reversal experiments consisted of minimal broth, minimal and supplemented broth containing trimethoprim and supplemented broth without trimethoprim. All tubes were incubated at 37 C for 18 to 24 hr.

Trimethoprim dose-response in various media. All media were prepared in double strength and diluted to single strength in the assay tubes. The final volume was 5.0 ml. Seed cultures of *E. coli* B were grown for 18 to 20 hr at 37 C in the same broth as that in which the test was performed. The inocula were standardized as described above. The 50 and 90% inhibitory concentrations of trimethoprim (IC_{50} and IC_{90}) are defined as that concentration of drug ($\mu\text{g}/\text{ml}$) which reduced growth to 50 and 10% of a control culture grown in the same medium but lacking trimethoprim. In experiments where known concentrations of thymidine were added to the medium, the thymidine was added to the tubes containing double-strength medium and autoclaved. The turbidity of the cultures was measured after 20 to 21 hr of incubation.

Assay for thymidine. This assay is based on the reversal by thymidine of the inhibition of growth of *Lactobacillus arabinosus* by 2,4-diamino-6,7-di-phenylpteridine. The pteridine inhibitor was synthesized as the hydrochloride in these laboratories (11) and dissolved in absolute methanol. The method followed was essentially as described by Lansford et al. (10) with the following modifications. The final concentration of inhibitor used in the assay was increased to 5.0 $\mu\text{g}/\text{ml}$ instead of the recommended level of 2.75 $\mu\text{g}/\text{ml}$. A strain of *L. arabinosus* carried in this laboratory was used as assay organism, with an inoculum concentration 10-fold higher than originally recommended. Inoculum stocks were washed twice with normal saline before use. The sensitivity of the modified assay was somewhat less than the assay originally reported, but values as low as 0.02 μg of thymidine could be determined reliably. Standard solutions of thymidine (0.01 to 2.0 μg) were included in each determination. Samples of media for assay were prepared in double distilled water at a concentration of 10 mg/ml except tryptic soy (Difco) and Trypticase soy broths (BBL) at 20 mg/ml. Duplicate tubes each received 1.0 ml of serial twofold dilutions (in double distilled water) of the sample being tested, and a second series of tubes received the same volume of sample plus thymidine at levels given in Table 2. The concentration of thymidine was calculated from

plots of the Klett readings at 660 nm after 18 hr of growth at 30 C. The thymidine concentration calculated for each medium represents an average of values obtained for 4 to 12 tubes.

Detection of mutants lacking thymidylate synthetase. *E. coli* B was grown in duplicate tubes in supplemented broth containing thymidine and trimethoprim at a concentration of 10 $\mu\text{g}/\text{ml}$ each. After 24 hr, the cultures were pooled, washed twice, and diluted in saline. A sample was spread on the surface of plates prepared from supplemented broth (plus 1.5% agar) containing thymidine (10 $\mu\text{g}/\text{ml}$). Over 500 colonies were picked with sterile applicator sticks from these plates to supplemented plates with and without thymidine. Colonies that appeared only on plates containing thymidine were subcultured to a second set of plates, as described above, to confirm their growth characteristics. Growth in the presence of thymidine but not in its absence was considered presumptive evidence of the loss of thymidylate synthetase.

RESULTS

Requirements for growth in the presence of trimethoprim. The inhibitory action of trimethoprim in minimal broth is shown in Fig. 1. Inhibition is evident at concentrations of trimethoprim below 0.05 $\mu\text{g}/\text{ml}$, and suppression of growth is complete at 1.0 $\mu\text{g}/\text{ml}$.

Table 1 shows the results of experiments in which attempts were made to reverse the inhibition produced in minimal medium by trimethoprim (10 $\mu\text{g}/\text{ml}$). In the absence of thymidine, no growth was detected in any medium. The maximum degree of reversal of inhibition, approximately 50% of control growth, was seen in the presence of thymidine and the complete supplement of amino acids, ribosides, and vitamins. A lesser degree of reversal was obtained in the absence of vitamins.

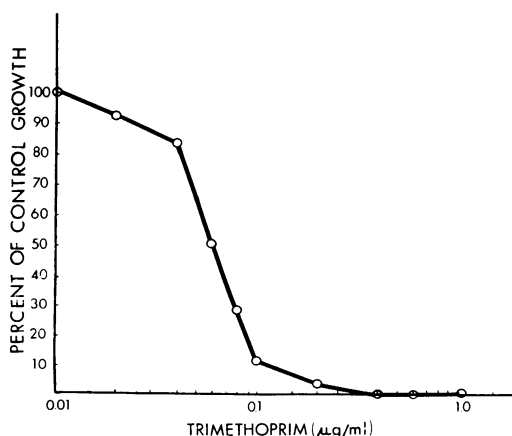


FIG. 1. Response of *E. coli* B to trimethoprim in minimal broth.

TABLE 1. Requirements for growth of *Escherichia coli* B in minimal broth in the presence of trimethoprim^a

| Supplements | Turbidity ^b | | |
|--|------------------------|---------------------------------------|---------------------|
| | Trimethoprim | | |
| | 0 $\mu\text{g/ml}$ | 10 $\mu\text{g/ml}$ plus thymidine at | |
| | | 0 $\mu\text{g/ml}$ | 10 $\mu\text{g/ml}$ |
| None..... | 95 | 0 | 0 |
| Amino acids..... | 124 | 0 | 0 |
| Amino acids, ribo- sides..... | 130 | 0 | 31 |
| Amino acids, ribo- sides, vitamins..... | 131 | 0 | 70 |

^a All data given are the average of two experiments. The composition of the amino acid, ribo-
side, and vitamin mixtures is given in the text.

^b Values are expressed as Klett units at 660 nm.

Response of *E. coli* B to thymidine. Figure 2 shows the effect of increasing concentrations of thymidine on the growth of *E. coli* B in supplemented medium containing trimethoprim (10 $\mu\text{g/ml}$). As little as 0.25 μg of thymidine per ml produced some growth response in the presence of trimethoprim (10 $\mu\text{g/ml}$). At higher levels of thymidine (2 $\mu\text{g/ml}$), maximum reversal was obtained. In other experiments, the inclusion of as little as 0.25 μg of thymidine per ml raised over 50-fold the quantity of trimethoprim needed for complete inhibition of *E. coli* B (from 1.0 $\mu\text{g/ml}$ to greater than 50 $\mu\text{g/ml}$).

Thymidine content and response of *E. coli* B to trimethoprim in commercially prepared media. The requirement for thymidine for the growth of *E. coli* B in the presence of trimethoprim suggested a possible correlation between the amount of thymidine in commercially prepared media and the degree of activity manifested by the inhibitor in these media. Samples of 12 different commercially prepared media were assayed for thymidine content in the presence and absence of added thymidine. The results of these assays and the IC_{50} and IC_{90} values determined for trimethoprim in each medium are shown in Table 2. The data in column 3 (thymidine, broth formula) give the content of thymidine in broth prepared by the manufacturer's directions.

The quantities of thymidine recovered range from less than 0.03 (Wellcome, nutrient broth) to 30.9 $\mu\text{g/ml}$ found in Brain Heart Infusion (BBL). The amount of thymidine measured in proteose peptone (Difco) was identical with that reported by Lansford et al. (10). It is clear that even

extremely low concentrations of thymidine decrease the sensitivity of *E. coli* B to trimethoprim; thus, the IC_{90} of trimethoprim in tryptic soy broth (Difco) was 1.8 $\mu\text{g/ml}$ as compared to 0.11 $\mu\text{g/ml}$ in minimal broth. When concentrations of thymidine greater than 1 $\mu\text{g/ml}$ were present, IC_{90} values for trimethoprim of greater than 50 $\mu\text{g/ml}$ were consistently observed.

To obtain a clearer idea of the relationship between thymidine content and degree of inhibition, the degree of inhibition of *E. coli* B was determined in the presence of trimethoprim (10 $\mu\text{g/ml}$) in each of the media listed in Table 2, and the results were plotted against the thymidine content of the broth. The results are seen in Fig. 3. It is clear from inspection of the figure that a correlation between the activity of trimethoprim and thymidine content exists up to a concentration of about 5 μg of thymidine per ml. Above this level of thymidine, no further reduction in the activity of trimethoprim is seen. Tryptone soy broth (Oxoid) seemed to give a lower degree of inhibition than would have been expected on the basis of its thymidine content. No explanation for this observation is apparent. Regression analysis of these data, omitting the data on the Brain Heart Infusion (BBL) which clearly contains a saturating level of thymidine, yields a correlation coefficient of 0.76. If the anomalous tryptone soy broth is omitted, the correlation coefficient increases to 0.86.

Thymidine requirement of *E. coli* B grown in the presence of trimethoprim and thymidine. Mutants lacking thymidylate synthetase (thymine-requiring) have been isolated from medium containing thymine and trimethoprim after pro-

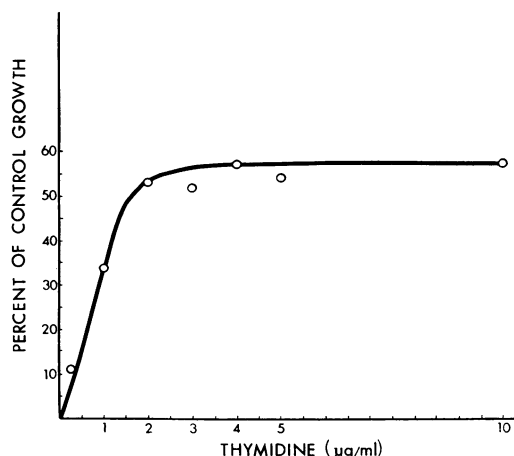


FIG. 2. Growth of *E. coli* B in increasing levels of thymidine. The medium is supplemented broth containing trimethoprim (10 $\mu\text{g/ml}$).

TABLE 2. *Thymidine content of commercially prepared media and the response of Escherichia coli B to trimethoprim*

| Sample | Thymidine added ($\mu\text{g/ml}$) | Thymidine recovered ($\mu\text{g/ml}$) | Thymidine in broth formula ($\mu\text{g/ml}$) | Trimethoprim ($\mu\text{g/ml}$) | |
|------------------------|---|---|---|--------------------------------------|------------------|
| | | | | IC ₅₀ | IC ₉₀ |
| Minimal broth | | | | 0.06 | 0.11 |
| Peptone | 0 | 3.86 | | | |
| (Difco, lot 438509) | 2.0 | 5.77 | | | |
| Proteose peptone | 0 | 5.09 | | | |
| (Difco, lot 510635) | 1.0 | 6.34 | | | |
| Nutrient broth | 0 | <0.01 | <0.03 | 0.09 | 0.17 |
| (Wellcome) | 1.0 | 0.83 | | | |
| Tryptic soy broth | 0 | 0.04 | 0.06 | 0.26 | 1.8 |
| (Difco, lot 511535) | 1.0 | 1.20 | | | |
| Sensitivity test broth | 0 | 0.02 | 0.07 | 0.42 | 0.8 |
| (Oxoid, lot 1105) | 1.0 | 1.04 | | | |
| Trypticase soy broth | 0 | 0.15 | 0.23 | 0.26 | 2.0 |
| (BBL, lot 109600) | 1.0 | 1.29 | | | |
| Mueller Hinton broth | 0 | 0.46 | 0.97 | 1.35 | >50 |
| (Difco, lot 513907) | 1.0 | 1.39 | | | |
| Tryptone soy broth | 0 | 0.44 | 1.32 | 50 | >50 |
| (Oxoid, lot 989) | 2.0 | 2.52 | | | |
| Antibiotic medium 3 | 0 | 0.85 | 1.49 | 0.9 | >50 |
| (Difco, lot 515762) | 1.0 | 2.05 | | | |
| Brain Heart Infusion | 0 | 0.79 | 2.92 | >50 | >50 |
| (Difco, lot 499064) | 2.0 | 2.67 | | | |
| Antibiotic assay broth | 0 | 2.76 | 4.83 | >50 | >50 |
| (BBL, lot 702620) | 1.0 | 3.90 | | | |
| Brain Heart Infusion | 0 | 8.37 | 30.9 | >50 | >50 |
| (BBL, lot 801628) | 1.0 | 9.36 | | | |

longed incubation (15). It was desirable to show whether decreased sensitivity to trimethoprim observed in the presence of end products of the folate pathway was due to the ability of these compounds to supply precursors of nucleic acids rather than to the selection of mutants with decreased requirements for tetrahydrofolate after loss of thymidylate synthetase (1).

Cultures of *E. coli* B were grown for 24 hr in supplemented broth containing thymidine and trimethoprim. A total of 567 colonies were examined (*see above*). Four colonies appeared to require thymidine for growth: an incidence of 0.7%. For this reason, it does not appear that the selection of thymidylate synthetase-negative mutants influenced the results of this study.

DISCUSSION

The introduction of new, synthetic antimicrobial agents requires increasing sophistication in *in vitro* susceptibility testing procedures so that the potential activity of the compounds will not be obscured by antagonists in the medium. Some of these antagonists act to compete with the inhibitor for the active site of a target enzyme, whereas others may noncompetitively bypass an

inhibited enzyme by supplying the end products of the target pathway. The ability of *p*-amino-benzoic acid to compete with sulfonamides for a position on the folic acid synthesizing enzymes is a well known example of the first situation (17), whereas the bypass of the inhibition of dihydro-folate reductase by thymidine illustrates the latter situation.

The possibility that a mechanism exists for the bypass of inhibition by trimethoprim was previously suggested by experiments in which thymidine was shown to counteract the activity of aminopterin against microorganisms (7, 14). However, it could not be assumed, in the absence of data, that these results could be generalized to the conditions under study in this paper. Aminopterin is an entirely different type of reductase inhibitor from trimethoprim. The former is a potent, toxic inhibitor which binds stoichiometrically to dihydrofolate reductase with no discrimination regarding the source of the enzyme (9, 16). Trimethoprim, on the other hand, binds to only a portion of the reductase normally occupied by dihydrofolate and shows a remarkable ability to distinguish among reductases from various species (2). The innocuous-

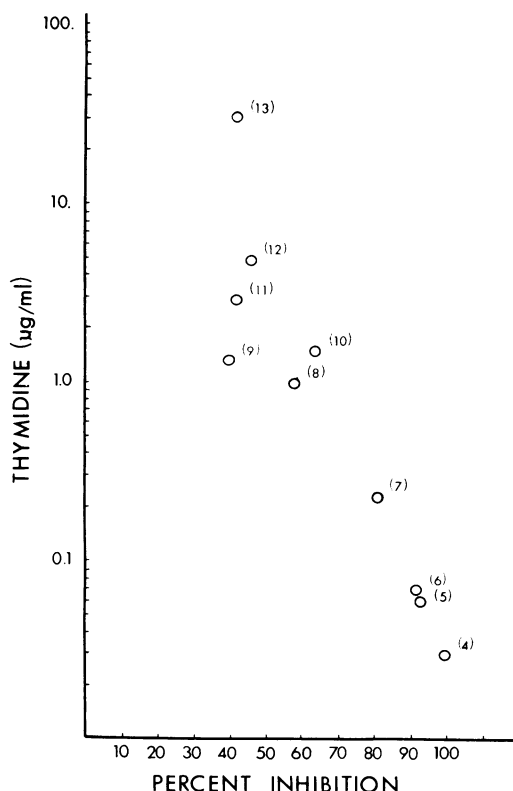


FIG. 3. Response of *E. coli* B to trimethoprim (10 µg/ml) in various commercially prepared media as a function of thymidine content. Concentrations of thymidine are given on a log scale on the ordinate. Media are numbered as in Table 2.

ness of the compound in humans is based on its preference for binding to bacterial rather than the mammalian reductase, the former enzyme being bound considerably more strongly than the latter.

In this study a correlation was seen between the concentration of thymidine in commercial media and the response of *E. coli* B to trimethoprim in these media. These studies point up the need for careful selection of media for sensitivity testing of bacteria to inhibitors such as trimethoprim. Trimethoprim showed greatest activity in nutrient broth (Wellcome). Sensitivity test broth (Oxoid) or tryptic soy broth (Difco) also allowed almost maximum expression of inhibition. Media of the same name from different manufacturers were not interchangeable as can be seen by a comparison of the thymidine levels in tryptic soy broth (Difco), Trypticase soy broth (BBL), and tryptone soy broth (Oxoid), and between antibiotic medium no. 3 (Difco

Penassay broth) and antibiotic assay broth (BBL).

Complete reversal of growth inhibition was not obtained despite the addition to the medium of compounds whose synthesis was known to be blocked. It is possible that not all of the potential reversing agents or combinations of reversing agents were supplied. A more probable explanation is based on the fact that formylmethionyl-transfer ribonucleic acid is required for the initiation of protein synthesis in *E. coli* (12). Trimethoprim, by blocking the formation of tetrahydrofolate cofactors, probably depletes the cell of formylmethionyl-transfer ribonucleic acid. Since this compound cannot be exogenously supplied to and assimilated by *E. coli*, it is not possible to bypass inhibition at this locus.

Finally, consideration should be given to the implications of these findings for the application to chemotherapy of inhibitors such as trimethoprim. If blood and body fluids contained substantial concentrations of thymidine, or its equivalent, growth of the microorganism could occur by the bypass mechanism, and the inhibitor would fail. A priori, this would not be expected, since sulfonamides are chemotherapeutically active, and the inhibition of folate biosynthesis is subject to a similar bypass reversal (9). Although the bodily pools of thymidine have not been established definitively, exogenously supplied thymidine has a limited half-life (8). In practice, trimethoprim exhibits substantial chemotherapeutic activity both alone and in combination with sulfonamides (18). For these reasons, those media which contain little or no thymidine appear more accurately to reflect the chemotherapeutic potential of the inhibitor than do those in which thymidine is present in the higher concentrations.

ACKNOWLEDGMENTS

We thank Gertrude B. Elion for synthesizing the pteridine inhibitor used in the thymidine assay. We also are grateful to William Shive for his helpful suggestions and G. H. Hitchings for encouraging and enlightening discussions.

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